The Improved Organ Maintenance of the Human Sebaceous Gland: Modeling *in Vitro* the Effects of Epidermal Growth Factor, Androgens, Estrogens, 13-*cis* Retinoic Acid, and Phenol Red

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We have previously reported that human sebaceous glands can be maintained for up to 14 d as whole organs with full retention of the physiological rate and pattern of new cell formation, but we have also reported that the newly formed cells did not differentiate normally, causing a progressive loss of lipogenesis *in vitro*. We now show that this abnormal sebocyte differentiation was attributable to the presence of epidermal growth factor (EGF) and phenol red in our maintenance medium. In their absence, human sebaceous glands apparently retain *in vivo* rates of cell division and lipogenesis over 7 d of maintenance in addition to a retention of *in situ* morphology. This is reversible on the re-addition of 10 ng EGF/ml and 10 mg phenol red/ml. The addition of 600 pM 17β-estradiol results in a significant fall in the rate of lipogenesis over 7 d of maintenance, without affecting the rate of cell division. This effect is apparently due to abnormal differentiation of newly formed sebocytes. Neither 1 nM testosterone nor 1 nM dihydrotestosterone (DHT) has any effect on rates of cell division of lipogenesis over 7 d. In the presence of phenol red, however, 1 nM testosterone or 1 nM DHT cause a significant reduction in the rate of lipogenesis over 7 d of maintenance. One micromolar 13-*cis* retinoic acid caused a significant reduction in the rate of lipogenesis over 7 d in both the presence and absence of phenol red. These findings show that we can model the physiological effects of steroids, EGF, and 13-*cis* retinoic acid *in vitro*. *Key word: steroid hormones. J Invest Dermatol 106:454–460, 1996*

The sebaceous gland *in vivo* demonstrates a number of responses to external stimuli. Androgens cause the gland to develop at puberty (Pochi *et al*, 1979), estrogens reduce the rate of sebum excretion in both humans (Strauss *et al*, 1962) and animal models (Ebling and Skinner, 1983), 13-*cis* retinoic acid will dramatically reduce sebaceous gland size and sebum excretion (Landhaler *et al*, 1980), while trauma (Irvin, 1981) and epidermal growth factor (Moore *et al*, 1985) promote an injury response.

To help understand the mechanism of these responses, we need to model them *in vitro*, and sebaceous glands maintained as whole organs should provide an optimal system. The whole gland on culture is small enough to permit diffusion of nutrients, gases, and growth factors to promote the optimal differentiation of sebocytes *in vitro*. Moreover, the culture of whole glands promotes the optimal differentiation of their cells *in vitro* as they develop within their appropriate environment (Freshney, 1987).

Earlier we showed that human sebaceous glands can be isolated by shearing (Kealey *et al*, 1986) and that freshly isolated glands demonstrate *in vitro* rates and patterns of lipogenesis that approximate those seen *in vivo* (Cassidy *et al*, 1986). These findings have been confirmed by others (Middleton *et al*, 1988). We have also shown that human sebaceous glands can be maintained for up to 2 wk in culture as whole organs (Ridden *et al*, 1990). During maintenance, rates of DNA and protein synthesis are maintained, but the rate of lipogenesis falls progressively. These findings indicate that, whereas normal rates of cell division are maintained in culture, the normal differentiation of newly formed cells into sebocytes is lost.

The medium we previously employed was supplemented with a large number of growth factors. To attempt to improve the organ maintenance of human sebaceous glands, we have now systematically excluded each supplement individually, and we now show that while the majority of supplements promoted lipogenesis on maintenance, epidermal growth factor (EGF) and phenol red, the pH indicator used in William's E medium, prevent normal sebocyte differentiation. In a new maintenance medium, we can apparently maintain the *in vivo* rate of lipogenesis and normal sebocyte differentiation over 7 d in organ culture. Using this improved model, we show that over 7 d of maintenance, 17β-estradiol at physiological concentrations and 13-*cis* retinoic acid at pharmacological concentrations significantly inhibit lipogenesis in the gland, while testosterone and dihydrotestosterone (DHT) at physiological concentrations have no apparent effect. Thus, apparently we can mimic the physiological actions of these compounds *in vitro*.

MATERIALS AND METHODS

William's E medium (with and without phenol red), L-glutamine, Fungizone, penicillin and streptomycin, and trace elements mix were from

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Gibco-BRL (Paisley, Scotland, U.K.). Tissue culture plastics were from Hawfell Scientific (Cambridge, U.K.). All other tissue culture reagents and chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.), or BDH Ltd. (Poole, Dorset, U.K.) and of the highest purity available. Polycarbonate filters were from The Nucleopore Corporation (Pleasonton, CA). Radiochemicals were from Amersham International, (Amersham, Bucks, U.K.).

Isolation of Viable Human Sebaceous Glands Samples of normal midline chest skin (5 × 80 mm) were obtained from male patients aged between 40 and 75 y undergoing cardiac surgery at Papworth Hospital, Papworth Everard, Huntingdon, U.K. Both ethics committee permission and patient consent had been granted for this technique. Glands were isolated by shearing (Kealey, 1990). Briefly, subcutaneous fat was removed with care being taken not to damage the dermal layer. Skin was washed in Earle's balanced salt solution; cut into small pieces about 5 mm in length, using sharp scissors, into 10 ml of Earle's balanced salt solution; and repeatedly cut until it reached a porridge-like consistency. This mixture was diluted with phosphate-buffered saline to give an approximate dilution of 1:4, Earle's balanced salt solution to phosphate-buffered saline. The glands could easily be seen under a dissecting microscope. Surrounding collagen was removed by careful microdissection.

Organ Maintenance of Sebaceous Glands Glands were maintained floating on polycarbonate filters (pore size 0.45 μm) in 5 ml of William's E medium at 37°C supplemented with 2 mM L-glutamine, 100 units/ml penicillin and streptomycin, 2.5 μg/ml Fungizone, 10 μg/ml insulin, 10 ng/ml EGF, 10 μg/ml transferrin, 10 ng/ml hydrocortisone, 10 ng/ml sodium selenite, 3 nM triiodothyronine, 1% (vol/vol) trace elements mix, 10 μg/ml bovine pituitary extract, and buffered in a humidified 5% CO_2/95% air atmosphere. In the text, this will be referred to as supplemented William's E medium. Where glands are maintained in supplemented phenol red–free William's E medium without the addition of EGF, this will be referred to in the text as new supplemented William's E medium. As well as acting as an indicator, phenol red also protects the medium from damage by light and hence phenol red–free William's E must be handled in the dark.

Where appropriate, 10% fetal bovine serum (FBS), 1 μm 13-*cis* retinoic acid, 1 nM testosterone, 1 nM DHT, or 600 pM water soluble 17β-estradiol was added. The concentrations of testosterone, DHT, and 17β-estradiol are all in the physiological range (Cook and Beastall, 1987). We chose 1 μM 13-*cis* retinoic acid because it is the clinical therapeutic concentration (Colburn and Gibson, 1985) and the lowest concentration that has previously given maximal inhibition of lipogenesis in the isolated sebaceous gland (Ridden *et al*, 1990). 13-*cis* retinoic acid was dissolved in dimethylsulphoxide, the final concentration of which did not exceed 0.02% (vol/vol), and testosterone and DHT were dissolved in ethanol, the final concentration of which did not exceed 0.002% (vol/vol). Where these additions were absent from control experiments, an equal volume of vehicle was added.

[U-^{14}C]Acetate Incorporation into Lipids Five sebaceous glands were incubated in a Eppendorf tube containing 300 μl of bicarbonate-buffered saline (Na^+, 144 mM; K^+, 5.9 mM; Mg^{2+}, 1.2 mM; Ca^{2+}, 1.25 mM; Cl^-, 125 mM; SO_4^{2-}, 1.2 mM; PO_4^{3-}, 1.2 mM; HCO_3^-, 25 mM, pH 7.4 (Krebs and Hensleit, 1932), containing 2 mM [U-^{14}C]sodium acetate (specific activity 833 μCi/mmol) in a humidified 5% CO_2/95% air atmosphere at 37°C, and the rate of radionucleotide uptake into lipids was determined as previously described (Ridden *et al*, 1990). Briefly, after 1.5 or 3 h of incubation, the glands were removed and washed in four changes of phosphate-buffered saline, and the lipids were extracted by homogenizing glands in a glass/glass homogenizer in chloroform:methanol:0.88% KCl: water, at a ratio of 2:2:1:0.8 (Bligh and Dyer, 1959). The chloroform fraction was filtered through glass wool and collected in a 20-ml glass scintillation vial. The extract was dried under a stream of nitrogen, and 10 ml of scintillant was added. The radioactivity was determined using a Tricarb system 4000 β counter. Control experiments showed the rate of lipogenesis to be linear over 24 h.

Lipid Class Identification by Thin-Layer Chromatography The dried sebaceous gland lipid extract was redissolved in 300 μl of chloroform: methanol:0.88% potassium chloride (5:5:1, by volume) and spotted onto a 20 × 20 cm, 250 μm silica gel chromatography plate, and developed as previously described (Ridden *et al*, 1990). Briefly, the dried sebaceous gland lipid extract was redissolved in 300 μl of chloroform:methanol:0.88% potassium chloride (5:5:1, by volume), spotted onto a 20 × 20 cm, 250 μm silica gel chromatography plate. The plate was then developed in four different directions in four different solvent mixtures (Cooper *et al*, 1974). Solvent 1 consisted of light petroleum 40–60°C:diethyl ether:acetic acid (50:50:1). Solvent 2 consisted of light petroleum 40–60°C:benzene (70:30). Solvent 3 consisted of light petroleum 40–60°C only. In order to separate out the polar lipids, the bottom 3 cm of the plate was cut off and developed in Solvent 4, which consisted of chloroform:methanol:acetic acid:water (25:15:4:2). Each plate was run with a lipid standard plate. The plates were autoradiographed using Kodak X-omat AR x-ray film, and the radioactive spots removed into scintillation vials and 10 ml scintillant added. The radioactivity was determined using a Tricarb system 4000 β counter.

Rates of DNA Synthesis Five glands were incubated in 500 μl William's E medium containing 3 μM [methyl-^{3}H]thymidine (specific activity 1.33 Ci/mmol) and equilibrated at 37°C in a humid 5% CO_2/95% air atmosphere for 1.5 or 3 h as previously described (Ridden *et al*, 1990). Glands were washed four times in phosphate-buffered saline containing 3 mM thymidine and then homogenized in 100 mM K/ethylenediamine tetraacetic acid, pH 12.4, with a glass/glass homogenizer until an even suspension was obtained. The homogenate was transferred to an Eppendorf tube and centrifuged for 15 min at 12,000*g*, to remove cell debris. The supernatant was removed and precipitated by the addition of 500 μl 20% (vol/vol) PCA and 1 μl 10% (wt/vol) bovine serum albumin. Samples were left for at least 2 h. The resulting precipitate was collected by being passed, under vacuum, through Whatman GF/C filters previously washed with 10 mM thymidine to reduce any nonspecific binding of [methyl-^{3}H]thymidine to the filter. The filters were then washed with 10 ml of ice cold 10% (wt/vol) trichloroacetic acid and 5 ml of ice-cold 5% (wt/vol) trichloroacetic acid, followed by 1 ml of ethanol:diethyl ether, 1:1. The filters were dried at 60°C in an oven, and the radioactivity was determined by liquid scintillation spectrometry using a Tricarb system 4000 β counter. Control experiments have shown the uptake of [methyl-^{3}H]thymidine into perchloric acid–precipitable material to be linear up to 24 h.

Light Microscopy Glands were fixed in phosphate-buffered saline containing 4.25% glutaraldehyde for 24 h. The glands were then processed by standard methods, followed by staining with hematoxylin and eosin.

[Methyl-^{3}H]Thymidine Autoradiography Glands were incubated in 500 μl William's E medium containing 5 μCi [methyl-^{3}H]thymidine (specific activity 49 Ci/mmol) for 24 h at 37°C in a humid 5% CO_2/95% air atmosphere. After incubation, glands were washed in four changes of phosphate-buffered saline containing 3 mM thymidine and processed as described for light microscopy. Autoradiographs were prepared using Ilford K5 dipping emulsion. Sections were stained with hematoxylin using standard methods.

RESULTS

All Supplements Are Necessary for Gland Maintenance with the Exception of FBS and EGF On isolation, glands demonstrated typical *in situ* morphology and the three distinct sebocyte cell types were seen: (i) peripheral undifferentiated cells; (ii) partially differentiated cells; and (iii) mature differentiated sebocytes containing many lipid droplets **(Fig 1*a*)**.

After 7 d of maintenance in supplemented William's E medium, there was a large increase in the number of undifferentiated peripheral cells within the lobule of the sebaceous gland **(Fig 1*b*)**. Mature sebocytes were only present centrally, and there was hyperkeratinisation of the luminal surface of the gland. In addition, we saw vacuoles in the acini of some glands that were not present in fresh glands. This may be artifactual; maintained sebocytes may be more susceptible to damage during histological processing. The rate of lipogenesis fell from 1131.5 ± 129.3 pmol/mg wet wt/h to 201.9 ± 38.6 pmol/mg wet wt/h **(Table I)**, which confirms our previous findings (Ridden *et al*, 1990).

All supplements had been previously added arbitrarily on the assumption that all were necessary for improved gland maintenance. To establish which of these supplements were essential, and to exclude those that had no effect or were inhibitory, we maintained glands with each of the supplements removed in turn, with all the others still present. Removing EGF resulted in increased rates of lipogenesis over 7 d **(Table I)**. In addition, the pattern of lipogenesis was very similar to that seen in freshly isolated glands **(Table II)**. This improvement was achieved without an increase in the rate of cell division. The subsequent, individual, removal of the other supplements from the medium, however, all resulted in lower rates of lipogenesis **(Table I)**.

Cassidy *et al* (1986) and Middleton *et al* (1988) have demonstrated higher rates of lipogenesis after overnight maintenance

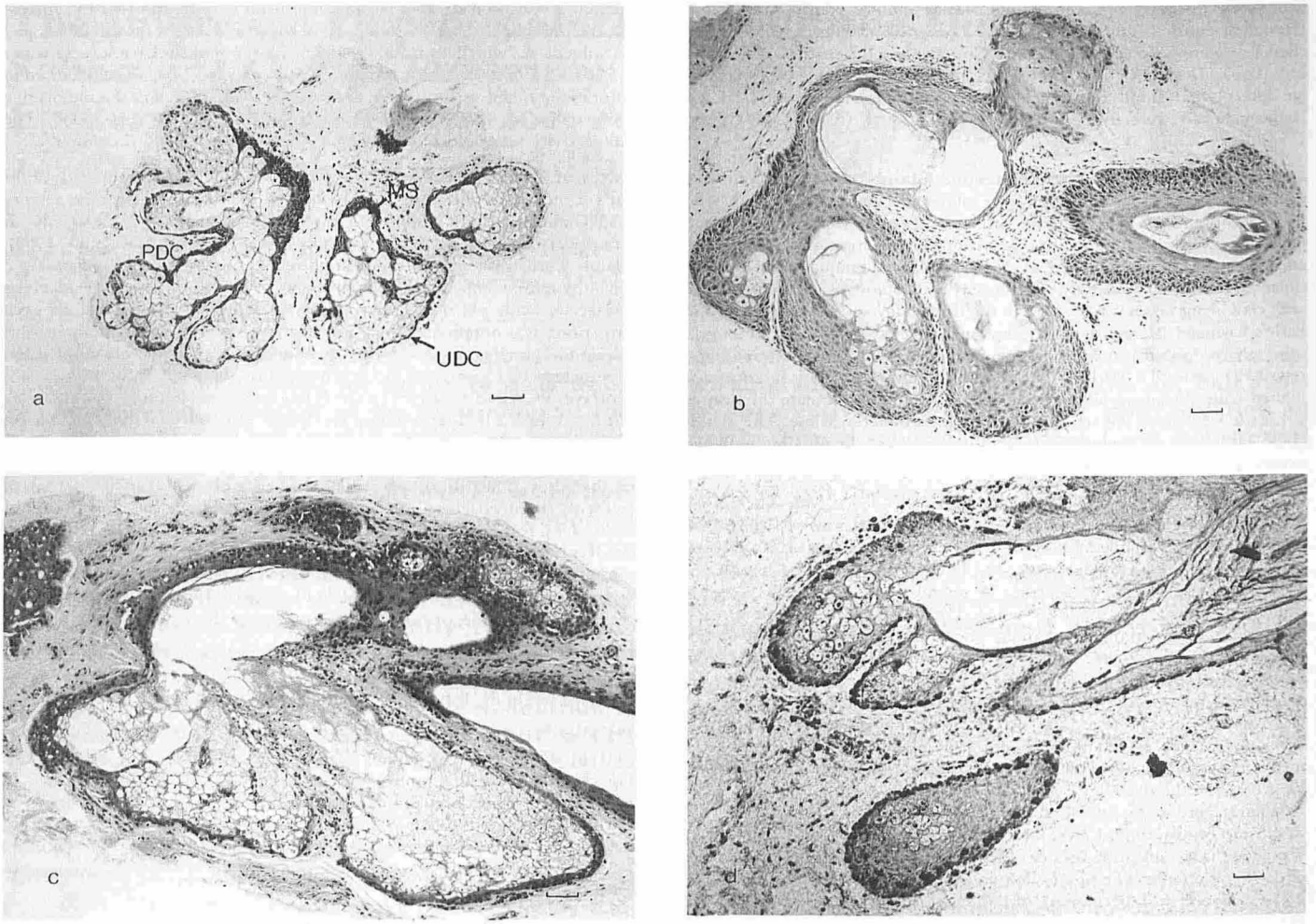

Figure 1. Sebaceous glands can be maintained with apparent retention of viability; EGF, 17β-estradiol, and 13-*cis* retinoic acid affect gland morphology. Glands were isolated by shearing and processed as described in *Materials and Methods*. *a*) A freshly isolated human sebaceous gland. The three distinct sebocyte cell types can be seen: (i) peripheral undifferentiated cells (*UDC*); (ii) partially differentiated cells (*PDC*); and (iii) mature differentiated sebocytes (*MS*) (representative of n = 5 subjects, five glands per subject). *b*) A sebaceous gland maintained for 7 d in supplemented William's E medium (representative of n = 5 subjects, five glands per subject). *c*) A sebaceous gland maintained for 7 d in new supplemented William's E medium (representative of n = 5 subjects, five glands per subject). *d*) Thymidine autoradiograph of a sebaceous gland maintained for 7 d in new supplemented William's E medium (without EGF and phenol red) (representative of n = 3 subjects, five glands per subject). *e*) A sebaceous gland maintained for 7 d in new supplemented William's E medium, further supplemented with 600 pM 17β-estradiol (representative of n = 5 subjects, five glands per subject). *f*) A sebaceous gland maintained for 7 d in new supplemented William's E medium, further supplemented with 1 nM testosterone (representative of n = 5 subjects, five glands per subject). *g*) A sebaceous gland maintained for 7 d in new supplemented William's E medium, further supplemented with 1 nM DHT (representative of n = 5 subjects, five glands per subject). *h*) A sebaceous gland maintained for 7 d in new supplemented William's E medium (without EGF and phenol red), further supplemented with 1 μM 13-*cis* retinoic acid (representative of n = 5 subjects, five glands per subject). *Scale bar*, 70 μM.

compared with rates immediately after isolation. Overnight maintenance may allow glands to recover from the trauma of isolation and hence result in increased rates of lipogenesis. For this reason, we maintained freshly isolated glands overnight before assaying. We have previously shown that the rate of lipogenesis for freshly isolated human sebaceous glands was 592.2 ± 60.3 pmols/gland/h (Ridden *et al*, 1990); however, those glands had been maintained overnight in William's E medium supplemented with both 10% FBS and 10 ng EGF/ml. We have shown that EGF inhibits lipogenesis after 1 wk of maintenance, and, since it has been reported that growth of cultured keratinocytes is enhanced in serum-free conditions (Boyce and Ham, 1983), we examined the effects of both EGF and FBS on the rate of lipogenesis in freshly isolated glands. When we maintained glands in supplemented William's E medium + 10% FBS, we found that rates of lipogenesis were significantly reduced compared with those in glands maintained in the absence of 10% FBS **(Table I)**. The removal of EGF from the maintenance medium did not cause a statistically significant increase in the rate of lipogenesis in glands maintained overnight.

It appears, therefore, that with the exception of EGF and FBS, all the other supplements are stimulatory and necessary for gland maintenance.

Testosterone, DHT, and 13-*cis* Retinoic Acid Inhibit Rates of Lipogenesis in Glands Maintained in Supplemented William's E Medium without EGF One nanomolar testosterone, 1 nM DHT, and 1 μM 13-*cis* retinoic acid all inhibited rates of lipogenesis over 7 d **(Table I)**. Furthermore, in the presence of 1 nM testosterone, the percentage of the total lipid synthesized accounted for by free fatty acids increased from 7.7 to 18.6% **(Table II)**, an effect not seen under the other conditions of maintenance. When glands were maintained in the presence of 1 nM DHT the percentage of total lipid synthesized by triglycerides fell from 12.4 to 4.3% and diglycerides rose from 2.8 to 10.7%

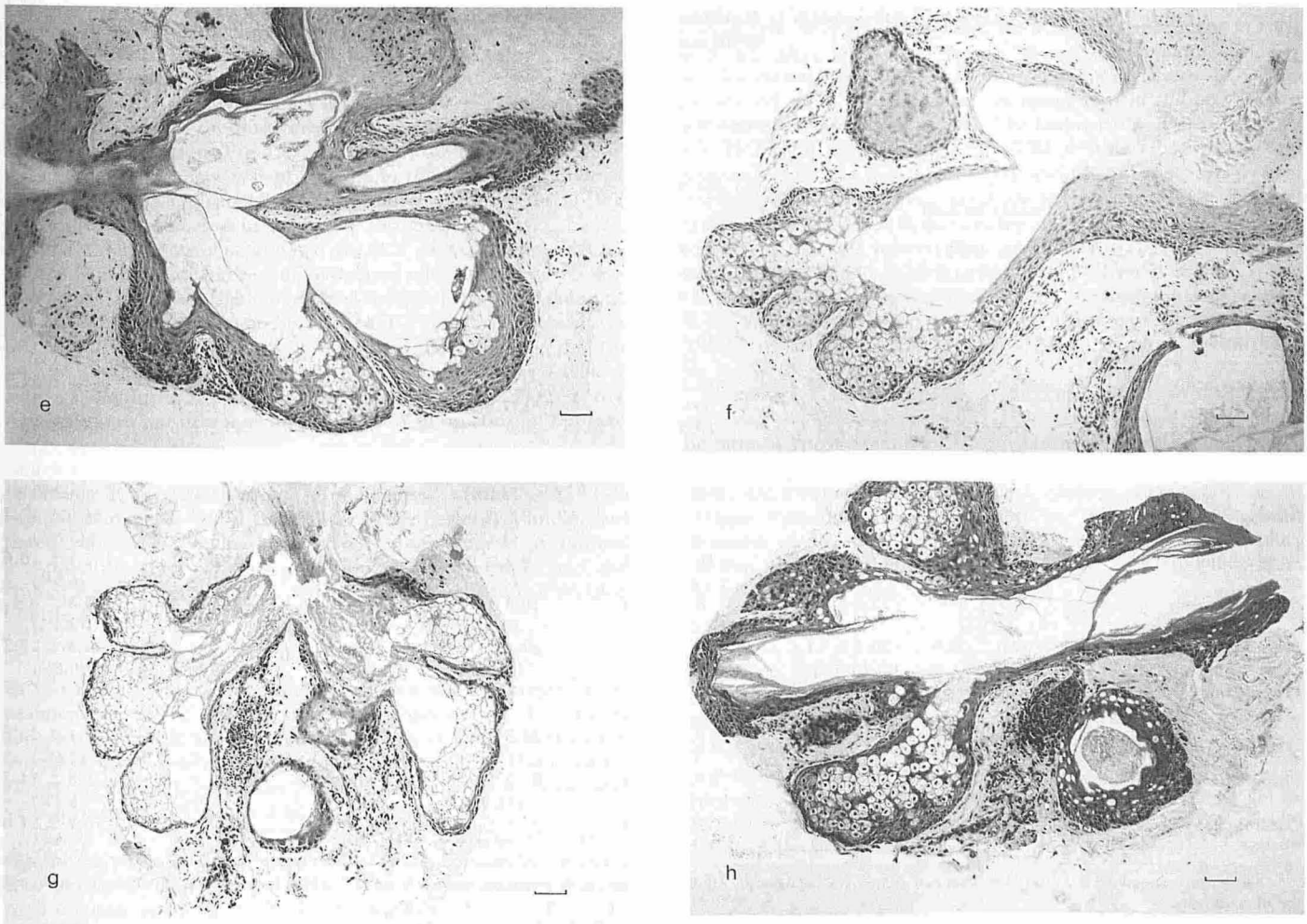

Figure 1. Continued

(Table II). Although results attained significance, their biological significance remains obscure.

Phenol Red–Free Medium Promotes Improved Gland Maintenance When glands were maintained for 7 d in new supplemented William's E medium (without phenol red and EGF) there was apparently almost perfect retention of *in situ* morphology **(Fig 1*c*)**. As with the fresh gland, the three distinct sebocyte cell types were seen. This suggests that normal gland function is maintained over 7 d in culture in this medium. In addition to the improvements in gland morphology, we found that maintaining glands in phenol red–free medium further improved rates of lipogenesis, such that

Table I. All Supplements to William's E Medium except FBS and EGF Are Necessary[a]

Condition of Maintenance	Rates of Lipogenesis (pmols/mg wet wt/h; mean ± SEM)		Rates of Cell Division (fmols/mg wet wt/h; mean ± SEM)	
	Fresh	7 d	Fresh	7 d
Supplemented William's E	1131.5 ± 129.3	201.9 ± 38.6[b]	—	—
Supplemented William's E + 10% FBS	837.9 ± 110.3	—	—	—
Supplemented William's E without EGF	1275.2 ± 117.0	643.2 ± 150.7[c]	168.2 ± 15.9	160.4 ± 17.2
Without T3 (n = 3)	—	352.6 ± 125.0[d]	—	—
Without transferrin (n = 3)	—	278.0 ± 51.6[d]	—	—
Without bovine pituitary extract (n = 3)	—	275.1 ± 17.2[d]	—	—
Without trace elements (n = 3)	—	237.3 ± 55.9[d]	—	—
Without hydrocortisone (n = 3)	—	196.6 ± 34.4[d]	—	—
Without sodium selenite (n = 3)	—	183.1 ± 21.5[d]	—	—
Without insulin (n = 3)	—	115.3 ± 19.4[d]	—	—
+1 nM testosterone	—	192.1 ± 25.2[d]	—	148.2 ± 10.1
+1 nM DHT	—	196.5 ± 30.9[d]	—	149.6 ± 11.9
+1 μM 13-*cis* retinoic acid	—	97.0 ± 13.9[d]	—	104.6 ± 8.4[d]

[a] Glands were maintained and assayed as described in *Materials and Methods*. n = 5 subjects for all conditions, five glands per subject per time point.
[b] Significant difference ($p < 0.01$) with regard to fresh glands maintained in supplemented William's E medium as determined by Student's paired t test.
[c] Significant difference ($p < 0.01$) with regard to fresh glands maintained in supplemented William's E medium without EGF as determined by Student's paired t test.
[d] Significant difference ($p < 0.01$) with regard to glands maintained for 7 d in supplemented William's E medium without EGF as determined by Student's paired t test.

Table II. The Pattern of Lipogenesis Is Retained in Glands Maintained for 7 d in New Supplemented William's E Medium[a]

	Supplemented William's E without EGF					New Supplemented William's E (without EGF and phenol red)	
	Freshly Isolated	7 d	+1 nM Testosterone 7 d	+1 nM DHT 7 d	+1 μM 13-*cis* Retinoic Acid 7 d	Fresh	7 d
Squalene	204.8 ± 65.1 (16.7%)	81.5 ± 32.7 (14.6%)	16.7 ± 6.7 (8.7%)	8.4 ± 1.7 (4.1%)[b,c]	5.2 ± 0.2 (5.4%)	253.9 ± 33.2 (16.5%)	245.3 ± 44.0 (16.6%)
Wax + cholesterol esters	119.3 ± 28.9 (9.4%)	46.4 ± 11.2 (8.3%)	9.9 ± 2.9 (5.1%)	6.6 ± 1.1 (3.3%)	4.2 ± 0.7 (4.3%)	143.1 ± 19.1 (9.3%)	133.6 ± 24.5 (9.1%)
Triglycerides	242.4 ± 40.5 (19.5%)	68.9 ± 21.2 (12.4%)	10.0 ± 2.1 (5.2%)	8.4 ± 2.3 (4.3%)[d]	8.2 ± 1.4 (8.5%)	297.0 ± 38.3 (19.3%)	277.9 ± 50.9 (18.9%)
Free fatty acids	91.8 ± 16.1 (7.2%)	42.8 ± 11.0 (7.7%)	35.7 ± 8.4 (18.6%)[c,d]	14.5 ± 4.3 (7.4%)	3.8 ± 0.6 (3.9%)	107.1 ± 14.1 (7.0%)	114.8 ± 21.1 (7.8%)
1,3-Diglyceride + 1,2-Diglyceride	43.3 ± 1.3 (3.4%)	15.5 ± 3.7 (2.8%)	10.5 ± 2.8 (5.5%)	20.9 ± 9.8 (10.7%)[c]	3.7 ± 0.9 (3.8%)	49.2 ± 6.3 (3.2%)	48.6 ± 8.4 (3.3%)
Cholesterol	39.5 ± 8.9 (3.1%)	16.6 ± 3.5 (3.0%)	4.4 ± 0.7 (4.3%)	7.8 ± 0.6 (4.0%)	3.8 ± 1.0 (3.9%)	52.4 ± 6.7 (3.4%)	47.1 ± 8.6 (3.2%)
Monoglyceride	48.5 ± 8.2 (3.8%)	23.2 ± 2.4 (4.1%)	9.8 ± 0.8 (5.1%)	8.0 ± 0.6 (4.1%)	3.5 ± 0.8 (3.6%)	62.0 ± 7.9 (4.0%)	56.0 ± 10.3 (3.8%)
Lysolecithin	91.8 ± 30.7 (7.2%)	17.3 ± 4.2 (3.1%)	16.1 ± 4.8 (8.4%)	9.4 ± 0.2 (4.8%)	9.3 ± 0.3 (9.5%)	101.5 ± 13.0 (6.6%)	102.1 ± 18.6 (6.9%)
Sphingomyelin	26.7 ± 5.4 (2.1%)	19.8 ± 4.6 (3.5%)	8.4 ± 1.0 (4.4%)	10.8 ± 1.3 (5.5%)	7.0 ± 0.6 (7.3%)	33.8 ± 4.3 (2.2%)	35.3 ± 6.4 (2.4%)
Phosphatidyl choline	127.5 ± 22.9 (10.0%)	50.4 ± 14.4 (9.0%)	17.2 ± 7.1 (9.0%)	27.4 ± 10.3 (14.6%)	5.8 ± 0.5 (5.9%)[c,d]	147.9 ± 19.0 (9.6%)	144.4 ± 27.2 (9.8%)
Phosphatidyl serine + inositol + ethanolamine	99.4 ± 20.8 (7.8%)	37.2 ± 20.5 (6.7%)	19.5 ± 4.9 (8.1%)	22.5 ± 1.7 (11.5%)	13.4 ± 3.3 (13.9%)	113.8 ± 14.5 (7.4%)	112.1 ± 20.6 (7.6%)
Phosphatidic acid	68.8 ± 14.1 (5.4%)	20.6 ± 2.2 (5.7%)	19.5 ± 4.9 (10.2%)	25.2 ± 4.9 (12.9%)	12.6 ± 4.0 (13.0%)	90.8 ± 11.5 (5.9%)	79.8 ± 14.3 (5.4%)
Cardiolipin	49.7 ± 16.3 (3.9%)	61.5 ± 10.1 (9.0%)	12.4 ± 4.8 (6.5%)	21.5 ± 6.4 (11.8%)	15.5 ± 1.9 (16.0%)[b]	86.2 ± 11.5 (5.6%)	76.7 ± 14.0 (5.2%)

[a] Glands were maintained and assayed as described in *Materials and Methods*. All data is expressed as pmols/mg wet wt/h (mean ± SEM); n = 3 subjects, five glands per subject for all conditions.
[b] Significant difference ($p < 0.01$) with regard to freshly isolated glands as determined by Student's t test.
[c] Significant difference ($p < 0.05$) with regard to glands maintained for 7 d in supplemented William's E medium without EGF as determined by Student's t test.
[d] Significant difference ($p < 0.05$) with regard to freshly isolated glands as determined by Student's t test.

they did not fall over 7 d in culture **(Table III)**. The pattern of lipid synthesis in glands maintained for 7 d in this new supplemented William's E medium was similar to that of freshly isolated glands **(Table II)**. There was, moreover, no change in the rate of cell division. Furthermore, when phenol red was returned to the medium, there was a reduction in the rate of lipogenesis to control levels **(Table III)**. In all subsequent experiments glands were maintained in supplemented phenol red–free William's E medium without EGF, and this is referred to in the text as new supplemented William's E medium.

The [methyl-^{3}H]thymidine autoradiographic pattern of glands maintained for 7 d in supplemented William's E medium without EGF or phenol red **(Fig 1*d*)** was identical to that of the isolated gland (Ridden *et al*, 1990) and the gland *in situ* (Epstein and Epstein, 1966; Plewig and Christophers, 1974) with radiolabel uptake taking place in the basal, undifferentiated cells of the gland.

Table III. Rates of Lipogenesis Are Maintained over 7 d in New Supplemented William's E Medium but Are Reduced by Phenol Red, 17β-Estradiol, and 13-*cis* Retinoic Acid[a]

	Rates of Lipogenesis (pmols/mg wet wt/h; mean ± SEM)		Rates of Cell Division (fmols/mg wet wt/h; mean ± SEM)	
Condition of Maintenance	Fresh	7 d	Fresh	7 d
New Supplemented William's E	1545.2 ± 283.9	1512.8 ± 175.4	154.9 ± 17.9	147.6 ± 18.4
+10 mg/ml phenol red	1361.7 ± 131.6	788.7 ± 140.4[b]	152.5 ± 19.5	149.9 ± 21.2
+600 pM estrogen	—	891.5 ± 153.1[c]	—	155.8 ± 33.2
+1 nM testosterone	—	1527.0 ± 163.1	—	145.1 ± 25.8
+1 nM DHT	—	1593 ± 181.3	—	151.4 ± 39.2
+1 μM 13-*cis* retinoic acid	—	783.8 ± 133.7[c]	—	94.1 ± 18.1[d]

[a] Glands were maintained and assayed as described in *Materials and Methods*. n = 5 subjects for all conditions, five glands per subject per time point.
[b] Significant difference ($p < 0.01$) with regard to glands maintained for 7 d in supplemented William's E without EGF and phenol red, and glands maintained for 7 d in supplemented William's E without EGF and phenol red further supplemented with 10 mg/ml phenol red, as determined by Student's paired t test.
[c] Significant difference ($p < 0.01$) with regard to glands maintained for 7 d in supplemented William's E without EGF and phenol red as determined by Student's paired t test.
[d] Significant difference ($p < 0.01$) with regard to glands maintained for 7 d in supplemented William's E medium without EGF and phenol red as determined by Student's paired t test.

17β-estradiol and 13-*cis* Retinoic Acid Affect Gland Maintenance over 7 d in New Supplemented William's E Medium, While Testosterone and DHT Have No Effect When glands were maintained for 7 d in new supplemented William's E medium, further supplemented with 600 pM 17β-estradiol, there was a thickening of the undifferentiated basal cell layer and some luminal keratinization **(Fig 1*e*)**. In addition, the rate of lipogenesis fell over 7 d, but there was no change in the rate of cell division **(Table III)**. This indicates that 17β-estradiol is altering the normal pattern of differentiation of the newly formed sebocytes.

When glands were maintained for 7 d in new supplemented William's E medium further supplemented with 1 nM testosterone, there was no apparent change in gland morphology **(Fig 1*f*)** and no change in the rate of lipogenesis **(Table III)**. Similarly, the addition of 1 nM DHT had no effect **(Fig 1*g*, Table III)**, indicating that the lack of an effect with testosterone is not simply due to a lack of active 5α-reductase in the gland. These findings suggest that androgens are not necessary for short-term stimulation of lipogenesis.

When glands were maintained for 7 d in new supplemented William's E medium further supplemented with 1 μM 13-*cis* retinoic acid, there was a thickening of the peripheral undifferentiated cells layer of the gland and a small degree of luminal keratinisation **(Fig 1*h*)**. One micromolar 13-*cis* retinoic acid also caused a significant fall in the rates of lipogenesis and cell division **(Table III)**.

DISCUSSION

In this study we have described significant improvements in the maintenance of the isolated sebaceous gland over 7 d in a new supplemented William's E medium. We have shown that in this improved model, where we are apparently able to retain normal sebaceous gland function over 7 d, we can model the *in vivo* actions of androgens, estrogens, and EGF.

The *in vitro* effects of EGF on the sebaceous gland may be significant, as there is extensive evidence for a significant role for EGF in skin development and maintenance. *In vivo*, human skin has high concentrations of the EGF receptor in the basal cells of the epidermis and in the skin appendages (Green and Couchman, 1985), and we have shown that EGF stimulates a population of dividing cells in the hair follicle (Philpott and Kealey, 1994). This and our own data suggest that EGF may play an important physiological role in sebocyte differentiation.

The *in vitro* effect of 17β-estradiol complements current *in vivo* data. Estrogens have been shown to inhibit lipogenesis in the human sebaceous gland (Strauss *et al*, 1962) and the rat preputial gland (Ebling and Skinner, 1983).

Phenol red has been described as having estrogen-like effects (Leake *et al*, 1987), which may explain its effects on gland morphology and lipogenesis, and why they are very similar to those of 17β-estradiol.

When we added testosterone to this improved model, we could not observe any effect. This was not due to a lack of 5α-reductase activity, since DHT was also found to have no effect. These *in vitro* effects apparently mimic those *in vivo*, as pharmacological doses of testosterone over a number of weeks in adult males cause only a small rise in the rate of sebum secretion (Strauss *et al*, 1962). In addition, the anti-androgen 17α-propyltestosterone has no effect on sebum excretion rate over 12 wk in humans (Franz *et al*, 1989), and it is only after much longer term treatment of up to 37 mo that sebum excretion rates are reduced with the anti-androgen cyproterone acetate (Burton *et al*, 1973). Therefore, while androgens are clearly essential for sebaceous gland development at puberty (Pochi *et al*, 1979) and for the long-term maintenance of sebum secretion, they do not appear to be necessary for short-term stimulation of lipogenesis.

When we maintained glands in new supplemented William's E medium with 10 mg/ml phenol red, however, and further supplemented this with physiological concentrations of testosterone or DHT, there was a significant fall in the rate of lipogenesis. This suggests that phenol red may modulate the actions of androgens, at least *in vitro*. EGF may also affect the actions of androgens *in vitro*, since we have previously shown that glands maintained in a supplemented William's E medium containing both EGF and phenol red show increased rates of lipogenesis in the presence of testosterone (Ridden *et al*, 1990). The biological significance of this is unclear and may be artifactual due to the suboptimal gland maintenance in the presence of EGF and phenol red; however, in other tissues steroids have been shown to affect EGF activity. Androgens and thyroid hormones are able to regulate EGF production in the murine submaxillary gland (Barthe *et al*, 1974), the submandibular gland (Gresik *et al*, 1981; Wilson *et al*, 1985), and the thyroid gland (Dagogo-Jack, 1992). Similarly, androgens decrease the level of EGF receptors in the prostate of the castrated rat (Traish and Wotiz, 1987). Thus, it is clear that androgens and EGF can interact.

The *in vitro* thickening of the undifferentiated layer of the gland seen with the addition of 13-*cis* retinoic acid, as well as the fall in the rates of lipogenesis, has been described *in vivo* (Landhaler *et al*, 1980; Jones *et al*, 1983) and demonstrates that we can apparently mimic the therapeutic action of 13-*cis* retinoic acid *in vivo*.

These data demonstrate that we can now maintain isolated sebaceous glands for 7 d in culture with full retention of viability and that we can model the physiological effects of steroids, EGF, and 13-*cis* retinoic acid.

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